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Exercise speeds cutaneous wound healing in high-fat diet-induced obese mice

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ABSTRACT

Purpose: Obesity has been shown to impair cutaneous wound healing which is associated with increased wound inflammation. Exercise is known to decrease obesity-associated inflammation and has been shown to speed cutaneous wound healing in aged mice. Therefore, we investigated whether treadmill exercise could speed cutaneous wound healing in obese, high-fat diet-fed mice. **Methods:** We fed female C57Bl/6J mice a high fat diet (45% calories from fat) for 16 weeks to induce a state of obesity and insulin resistance. Mice then ran on a treadmill for 3 days prior to excisional wounding. On day 4, mice were wounded 1h after exercise. Mice then exercised for 5 days post-wounding, and healing was assessed by photoplanimetry for 10 days. **Results:** As described previously, obesity impaired wound healing, with significantly larger wound sizes measured from days 3 to day 10 post-wounding ($p<0.05$). Exercise did not improve healing in lean mice fed a normal chow diet. However, wound size was significantly smaller in exercised obese mice compared to their lean counterparts ($p<0.05$ at day 1, day 4, day 5 post-wound). Surprisingly, we were unable to detect any differences in gene or protein expression of pro-inflammatory cytokines IL-1β and TNF-α or the anti-inflammatory cytokine IL-10 in the wounds. Likewise, there were no differences in gene expression of chemokines MCP-1 and KC or of growth factor PDGF in wounds of exercise and sedentary mice. **Conclusion:** This suggests an effect of exercise independent of alterations in inflammation. Future work should focus on early events post-wounding, including exercise effects on hemostasis and myofibroblast function. **Key words**: obesity, training, inflammation, mice

Obesity is a major health concern in the United States (US). From 13.4% prevalence in 1962, the percentage of US adults considered obese reached 30.9% in the year 2000 (8). This trend has continued, as the most recent national data indicates that nearly 34% of Americans can be considered obese, with more than 30% of the remainder now classified as overweight (7). Obesity and obesity-related disorders account for more than 300,000 deaths per year in the US alone (1, 13) and are estimated to be responsible for nearly $150 billion in health-care costs per year in the United States (6).

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Obesity and obesity-associated disorders such as cardiovascular disease (25), infectious disease (20), metabolic syndrome/diabetes (26), cancer (17, 29), and chronic kidney disease (14) are known to involve a dysregulation of the immune response including upregulated inflammation (9, 10) at both the systemic and local adipose tissue levels. This aberrant inflammatory response is associated with impairments in wound healing in obese individuals (38). This includes delays in healing of acute wounds and failure to heal chronic wounds; the latter of which leaves the individual prone to infection and other complications. Type 2 diabetes, a frequent comorbidity of obesity, is a leading cause of amputations in the US annually (33).

A number of studies have shown that delayed healing in animal models of obesity is associated with dysregulated inflammation. In genetically-obese mice, delayed healing has been shown to be associated with increased wound levels of pro-inflammatory chemokines (37) and cytokines (12).

Moreover, depletion of inflammatory cells including neutrophils (2) and macrophages (12) as well as treatment with neutralizing antibodies against pro-inflammatory cytokines such as tumor necrosis factor-α (12) have been shown to speed healing in genetically-obese animal models. Delayed healing may be mediated by prolonged induction of inflammation through macrophage dysfunction, as wound-associated macrophages in obese animals have been shown to fail to perform essential functions such as clearance of apoptotic neutrophils (22); a process that is necessary to switch the macrophage from a pro-inflammatory phenotype to an anti-inflammatory, pro-healing phenotype. Thus, interventions that target increased wound-associated inflammation and speed healing in obese individuals are of major public health and research interest.

Exercise has been shown to decrease inflammation in obese individuals at both systemic (4, 30) and adipose tissue-specific (35) levels. However, no studies to date have examined the effects of exercise training on healing rate or wound inflammation using an obesity model of delayed healing. Interestingly, exercise has been shown to speed healing in aged mice (21) and older humans (3) and reduce inflammation in the wounded tissue of aged mice (21). As aging causes increases basal levels of inflammation similar to that seen in obese individuals (23), similar mechanisms might allow exercise to speed healing in obese and diabetic individuals.

Therefore, the goal of this study is to determine the effects of exercise on the wound healing process in obese mice. We hypothesized that exercise would speed healing rate in obese mice similar to that shown in aged mice, and that this would be associated with a reduced level of inflammation in the wound tissue as in our previous aging study (21). A diet-induced obesity (DIO) model in which obesity is induced by feeding of a high-fat diet (45% kcal from fat) was chosen as evidence suggests that DIO models are most appropriate for studies of acute wounds while genetically-obese models are most appropriate for chronic, non-healing wound models (32). The intent of the exercise model used in this study as well as in the Keylock *et al.* study in aged mice (21) is to determine if a short-duration exercise training program performed within a known time frame prior to and after wounding can speed healing and lessen wound inflammation. The exercise paradigm and time points for the analyses described both in Keylock *et al.* (21) and in the present study are intended to contrast similar studies which show an impaired healing in mice subjected to restraint stress (19).

This intervention is targeted as a type of "prehabilitation" for obese individuals who are scheduled to undergo procedures such as bariatric surgery that will create cutaneous wounds. That is, should exercise be shown to speed healing rates in obese humans in future studies, short-term exercise training such as that described in this study may be prescribed to patients who will be undergoing surgery in order to help their wounds to heal faster post-surgery. As wounds from such procedures are known to heal more slowly in obese individuals (38), results from this pre-clinical study may have clinical potential.

METHODS

Experimental Animals Six-week old female C57Bl/6J (Jackson Labs, Bar Harbor, ME) were maintained individually in standard wire-top cages in an AAALAC-accredited animal care facility for 1 week prior to the start of experiments. Mice were allowed *ad libitum* access to water and to the diets. The mice were housed on a 12 hour reverse light-dark cycle with the dark period from 1000 to 2200 hrs. High-fat diet-fed mice were housed for 16 weeks and given an experimental diet (HFD) consisting of 45% total kcal from fat (Research Diets, New Brunswick, NJ). Food intake and body weight were measured every two weeks during the feeding portion of the study. In addition, food intake and body weight were assessed at day -3, day 0 (when wounds were applied), and day 6 during the exercise portion of the study. For characterization of the exercise effect on healing kinetics in chow-fed mice, 20-week-old female C57Bl/6J mice (Jackson Labs, Bar Harbor, ME) were maintained as described above for 4 weeks prior to wounding. The 4 groups hereinafter are referred to as HFD-Exercise (HFD-Ex), HFD-Sedentary (HFD-Sed), Chow-Exercise (Chow-Ex), and Chow-Sedentary (Chow-Sed). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Exercise Procedure Mice in the exercised groups ran on a motorized treadmill (Jog-a-Dog,

Ottawa Lake, MI) in individual lanes at 12 m/min and 5% incline for the final 30 minutes of the light period (0930-1000 hrs) starting at 3 days (Day -3) prior to wounding and continuing until 5 days (Day 5) post-wounding for a total of 9 days of exercise. Gentle prodding was used to encourage mice to continue running. This exercise protocol is similar to that previously used by our lab to study wound healing kinetics with exercise in aged mice (21). Non-exercised mice remained sedentary during this time but were exposed to noise and handling similar to that of the exercisers.

Wounding Procedure The wounding procedure, which results in two full-thickness excisional cutaneous wounds, is well-established and has been used in our lab in a previous study (21). Briefly, mice were anesthetized with isoflurane in oxygen at a rate of 2-3 $L \cdot min^{-1}$ starting at one hour after exercise on day 0. While anesthetized, mice were shaved and treated with three alternating scrubs of povidone-iodine (Purdue Pharma, Stamford, CT) and isopropyl alcohol. The skin was then folded over and excised using a 6.0mm diameter sterile, disposable punch biopsy instrument (HealthLink, Jacksonville, FL) to create two full-thickness dermal wounds. Mice were immediately photographed for assessment of baseline wound size and then returned to their home cage to recover. Return to consciousness after isoflurane administration takes about 30 seconds using this method. Mice were monitored until full recovery from the wounding procedure. No post-surgical pain medication, bandaging or hemorrhage control was provided in order to avoid any potential effects of such treatment on healing rate.

Analysis of Wound Closure Mice were photographed each day from Day 0-10 post-wounding in order to assess wound area. Briefly, mice were anesthetized with isoflurane administered with oxygen at a flow rate of 2-3 $L \cdot min^{-1}$ and wounds were photographed prior to recovery from anesthesia. Wound size was analyzed by photoplanimetry as previously described (21). Wound photograph files were re-coded by an investigator otherwise uninvolved with the study for blinding purposes prior to analysis.

Analysis of Gene and Protein Expression Mice used for gene and protein expression analysis were run in the same manner as described and were euthanized at day 1, day 3, or day 5 post-wounding. Mice were euthanized via rapid CO_2 asphyxiation and wounds were immediately harvested using an 8mm punch biopsy needle (Healthlink, Jacksonville, FL) and frozen on dry ice. Frozen wound tissue was stored at -80°C until processing.

Total RNA was isolated from frozen tissue using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA purity was assayed using an automated micro-volume Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA). RNA samples with 260/280 ratios near 2.0 were used for further analyses. Isolated RNA was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. The resulting cDNA was stored at -20°C until real-time polymerase chain reaction (RT-PCR) analysis for gene expression of selected inflammatory markers. RT-PCR analysis was performed on a high-throughput real-time PCR machine (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. All samples were run in duplicate.

Cytokines and chemokines tested for gene expression were interleukin (IL)-1β, IL-10, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC). Gene expression of platelet-derived growth factor (PDGF) was also assessed. TaqMan (Applied Biosystems, Carlsbad, CA) master-mix reagent and commercially-available, pre-validated primer-probe sets (Applied Biosystems, Carlsbad, CA) were utilized for all analyses. Expression of β-actin was used as the housekeeping gene, and expression of all genes was related to the housekeeping gene and to the referent (HFD-Sed Day 1) group by the $2^{-\Delta\Delta Ct}$ method (39).

Wound protein analyses of cytokines IL-1β, IL-10, and TNF-α were carried out by enzyme-linked immunosorbant assay (ELISA). Briefly, wounds were homogenized in ice-cold phosphate-buffered saline (PBS) with 0.5% v/v protease inhibitor (Sigma-Aldrich, St. Louis, MO) and centrifuged at 10,000×*g* to remove insoluble material. Supernatant was removed and assayed for total protein concentration by a commercially-available protein assay (DC Protein Assay, Bio-Rad, Hercules, CA). Protein was then stored at -20°C until analysis. Commercial ELISA matched antibody sets (murine

IL-10, R&D Systems, Minneapolis, MN; murine IL-1β and murine TNF-α, Peprotech, Rocky Hill, NJ) were used for all analyses. Dilutions for protein samples were as follows: IL-1β, 1:3; IL-10, no dilution; TNF-α, 1:1. All samples were diluted in PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO).

ELISAs were performed according to manufacturer's instructions with the following modifications. For the IL-1β and TNF-α assays, streptavidin-conjugated horseradish peroxidase (Strep-HRP, #890803, R&D Systems, Minneapolis, MN) was substituted for the provided Strep-HRP reagent and diluted 1:200 for use. For all assays, color development was carried out using a commercial substrate reagent set (BD OptEIA TMB Substrate Reagent Set, BD Biosciences, San Diego, CA), and reactions were stopped with 2*N* H_2SO_4. Color development was read at 450 nm on a spectrophotometric plate reader (BioTek, Winooski, VT). Protein concentrations are expressed relative to total protein isolated from each wound sample (pg cytokine per mg total protein).

Glucose Tolerance Testing Mice were assessed for insulin sensitivity via intraperitoneal (i.p.) glucose tolerance testing (GTT). GTT was performed on a subset of mice in each group 1 week prior to the exercise intervention as previously described (35). Mice were fasted overnight prior to GTT. At time 0, blood glucose was measured by tail nick using an automated glucometer (Johnson & Johnson, Langhorne, PA) after which mice were given 1g/kg glucose i.p. Blood glucose was similarly measured at 30, 60, 90, and 120 minutes post-injection. For the tail nick, a small portion of the tip of the tail was cut off using scissors, and blood (1-2 drops) was collected. Clotting takes place within less than 2 minutes post-nick, and only a small amount of blood is lost. GTT values were compared between HFD- and Chow-fed groups as a marker of metabolic derangement induced by the HFD. In order to determine if exercise-induced alterations in glucose tolerance were related to wound healing, a subset of mice underwent GTT at day 1 post-wounding in addition to the baseline testing. GTT was performed after an overnight fast, 24h after the previous exercise bout.

Statistical Analysis All results are expressed as mean ± SEM. Significance level was set at $\alpha = 0.05$. Body weight and food intake differences in HFD-fed and Chow-fed mice were assessed by unpaired *Student's* t-tests. Changes in body weight during the exercise intervention was analyzed by paired t-tests. Glucose tolerance tests were compared by repeated measures analysis of variance (ANOVA) with Tukey's HSD post-hoc analysis. GTT differences were also expressed as a comparison of area under the glucose response curve (AUC). Repeated-measures ANOVA (time × treatment – activity or diet) were used to analyze wound healing kinetics from Days 0-10 and or from Days 0-5 post-wounding. Gene and protein expression levels were analyzed by a 3×2 factorial ANOVA (day × activity). GTT response to exercise was analyzed by a 2×2 factorial ANOVA (time point × activity). In the instance of a significant interaction or treatment main effect, mean separation was carried out using Tukey's HSD post-hoc analysis. All analyses were carried out using SPSS v. 19.0 software (IBM, Somers, NY).

Table 1: Body weight and food intake for HFD and Chow mice

	HFD	Chow
Weight (g)	30.4 ± 0.7	23.7 ± 0.4*
Food (g·day^{-1})	3.1 ± 0.6	4.0 ± 0.1*
Energy (kcal·day^{-1})	14.7 ± 2.8	12.0 ± 0.3*

Weight: body weight at week 16 prior to exercise intervention. Food: average food intake per day. Energy: average kcal intake per day. HFD: high-fat diet-fed mice. Chow: chow diet-fed mice. All values are mean ± SEM. * Significant difference, HFD vs. Chow.

RESULTS

Body Weight and Food Intake By week 16 of the feeding intervention, HFD body weights were significantly higher than those of the chow group, as expected (Table 1). Food intake in grams per day was significantly lower in HFD compared to chow animals (Table 1). However, when expressed as energy intake (kcal per day), HFD had a greater energy intake compared to Chow animals, which explains their greater weight gain after 16 weeks of the study. There were no differences between exercised and sedentary mice in body weight or food intake, and the short (e.g. 9 day) exercise intervention did not result in significantly different food intake or weight loss compared to sedentary controls in either diet intervention (data not shown). In mice utilized for wound cytokine

Table 2: Body weight and food intake for HFD-Ex and HFD-Sed mice used for wound cytokine analysis.

	Weight Pre (g)		Weight Post (g)		Daily Food (g)	
Day	**HFD-Ex**	**HFD-Sed**	**HFD-Ex**	**HFD-Sed**	**HFD-Ex**	**HFD-Sed**
Day 1	30.1 ± 0.6	30.4 ± 1.2	29.2 ± 0.7*	30.0 ± 1.3	3.1 ± 0.1	3.2 ± 0.1
Day 3	31.6 ± 1.2	29.3 ± 1.6	30.6 ± 0.8	28.7 ± 1.2	3.3 ± 0.3	3.2 ± 0.1
Day 5	29.3 ± 1.3	30.5 ± 2.2	28.2 ± 1.4	29.8 ± 1.9	3.0 ± 0.2	2.9 ± 0.1

HFD: high-fat diet-fed mice. Ex: exercised mice. Sed: sedentary mice. Day indicates day of euthanasia. HFD-Ex: day 1 N=18, day 3 N=8, day 5 N=7; HFD-Sed: day 1 N=17, day 3 N=8, day 5 N=8. Pre: body weight at day -3 (onset of exercise). Post: body weight at day of euthanasia. All values are mean ± SEM. * Significant difference, pre vs. post body weight in the same treatment group (p=0.007).

analysis, there was a significant reduction of body weight in HFD-Ex mice euthanized at day 1, although the magnitude of the weight loss was small (0.9 g) and similar to non-significant changes in HFD-Ex mice euthanized at other time points as well as in HFD-Sed mice euthanized at all time points (Table 2). There were no significant differences in pre or post body weight or in food intake either between groups (Ex vs. Sed) or within groups at different time points (Table 2).

Glucose Tolerance Testing Glucose tolerance testing was performed 1 week prior to the wounding (week 15 of feeding) on a subset of HFD-fed (N=10) and chow-fed (N=5) mice. As expected, HFD-fed mice had an impaired response to glucose injection (Figure 1A), indicating a higher degree of insulin resistance in these mice (diet × time interaction $F_{1.9,24.3}$=4.794, p=0.019; diet main effect $F_{1,13}$=8.732, p=0.011). Analysis of area under the glucose response curve (AUC) also indicated that HFD-fed mice had impaired glucose tolerance (HFD AUC: 39132 ± 2445; Chow AUC: 28422 ± 1244; p=0.002). There were no differences between HFD-Ex and HFD-Sed mice at the 15 week baseline GTT measure (data not shown).

On a subset of mice (N=5 HFD-Ex; N=5 HFD-Sed), GTT was measured at day 1 post-wounding in addition to baseline (Figure 1B). Although HFD-Ex had slightly higher glucose intolerance at baseline (prior to exercise), there was no significant interaction (activity × time point $F_{1,8}$=0.160, p=0.699) nor were there significant time point (baseline vs. day 1, $F_{1,8}$=3.072, p=0.188) or activity (Ex vs. Sed, $F_{1,8}$=2.971, p=0.123) main effects, indicating that the exercise intervention had little effect on insulin resistance.

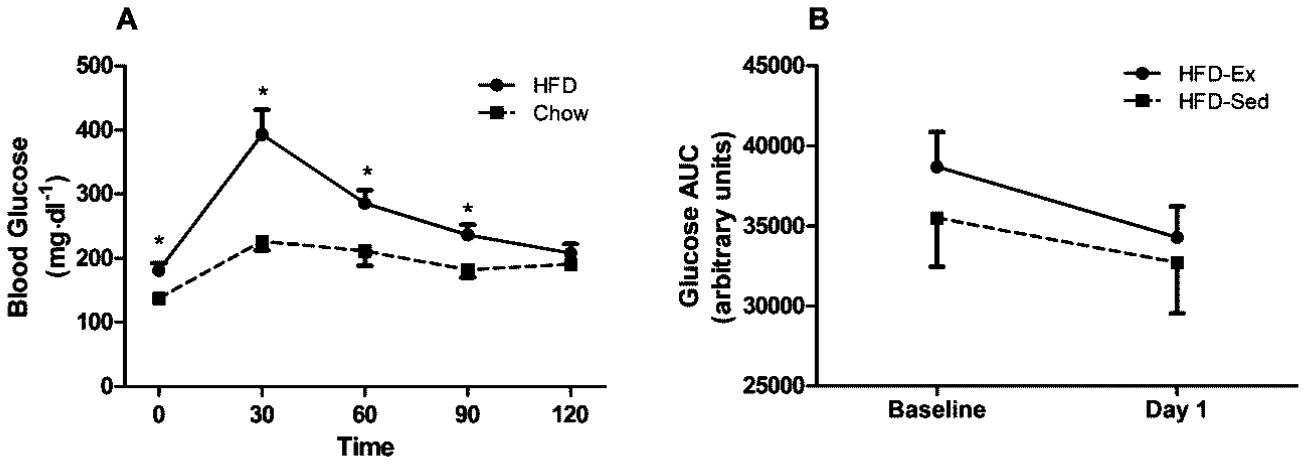

Figure 1: Glucose-tolerance testing. **A)** Glucose response in HFD- vs. Chow-fed mice prior to exercise. * Significantly higher blood glucose value in HFD vs. Chow mice at the same time point. HFD: high-fat diet-fed mice (N=10). Chow: chow diet-fed mice (N=5). **B)** Area under the glucose response curve prior to exercise and at day 1 post-wounding (after 4 days of exercise) in exercised and sedentary HFD-fed mice. HFD-Ex: exercised high-fat diet-fed mice (N=5). HFD-Sed: sedentary high-fat diet-fed mice (N=5). AUC: area under the glucose response curve. All values are mean ± SEM.

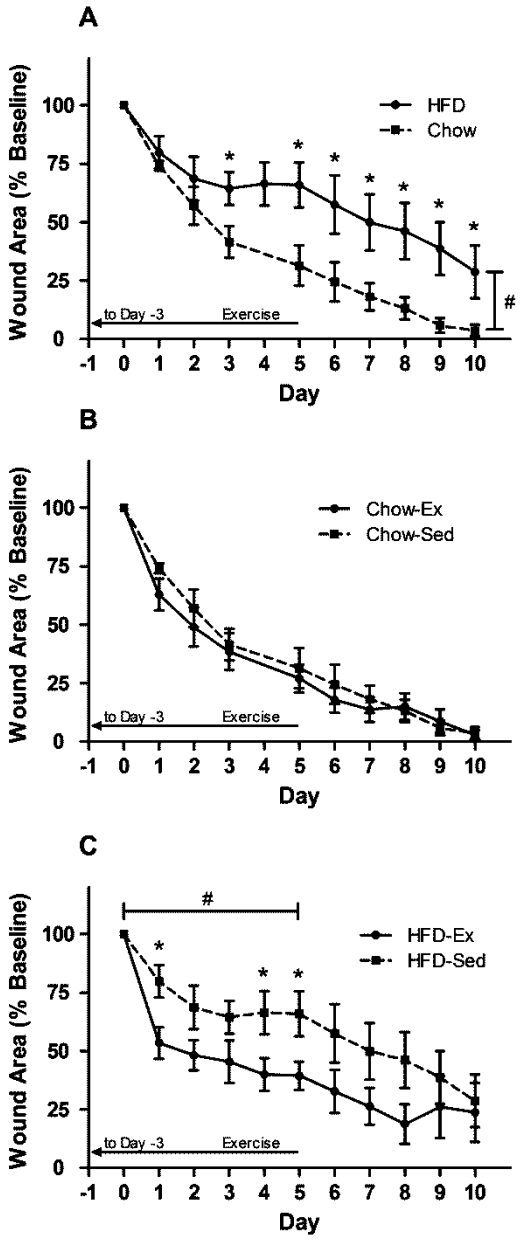

Figure 2: Wound healing kinetics in response to diet and exercise. All wound sizes are expressed as a percentage of baseline wound area (day 0). **A)** HFD-fed vs. chow-fed mice. # Significant time × diet interaction (day 0 through day 10, p=0.021). * Significant difference in wound size between HFD-fed and chow-fed mice at same day (p<0.05). HFD: high-fat diet-fed mice (N=7). Chow: chow diet-fed mice (N=8). **B)** Effect of exercise on healing kinetics in chow-fed mice. Chow-Ex: exercised chow-fed mice (N=8). Chow-Sed: sedentary chow-fed mice (N=8). **C)** Effect of exercise on healing kinetics in high-fat diet-fed mice. # Significant main effect of exercise (day 0 through day 5, p=0.050). The day × exercise interaction approached significant (p=0.053). * Significant difference in wound size between exercised and sedentary mice at same day (p<0.05). HFD-Ex: exercised high-fat diet-fed mice (N=6). HFD-Sed: sedentary high-fat diet-fed mice (N=7). All values are mean ± SEM.

Wound Closure As in previous studies (27, 28), high-fat diet feeding slowed wound healing rate (Figure 2A). When comparing wound healing kinetics in sedentary HFD-fed and Chow-fed mice for 10 days post-wounding, there was a significant time × diet interaction ($F_{2.9,39.4}$=3.655, p=0.021) and significant main effects for both diet ($F_{1,13}$=6.495, p=0.024) and time ($F_{2.9,39.4}$=59.955, p=0.000). Post-hoc analysis revealed that Chow-fed mice had significantly smaller wounds starting at day 3 and continuing to day 10 post-wounding (Figure 2A, p<0.05). We also tested whether exercise training could speed healing in both Chow-fed and HFD-fed mice. There was no additional effect of exercise on wound healing in Chow-fed mice (Figure 2B, time × activity interaction $F_{3.1,43.1}$=0.690, p=0.567). In HFD-fed mice, RM-ANOVA indicated a non-significant time × activity interaction (Figure 2C, $F_{3.0,33.4}$=1.669, p=0.192) but a main effect of activity which trended towards significance (Figure 2C, $F_{1,11}$=3.267, p=0.098). It should be noted that Chow and HFD mice in Figure 2A are identical to Chow-Sed and HFD-Sed in, respectively, Figures 2B and 2C. We have arranged the figure in this fashion as we have found that display of the figure in a 2×2 factorial arrangement obscures some of the important comparisons which we have highlighted here.

However, in our previous study in aged mice (21), the major effect of exercise was seen early (within 5 days) post-wounding. A qualitative evaluation of our healing data suggested that this might have been the case in the present study as well, as the differences in wound size between HFD-Ex and HFD-Sed were greater early (e.g. day 1-5) compared to late (e.g. day 8-10). Because of this, and because our original hypothesis was exercise would alter wound inflammation early post-wounding, we analyzed the effect of exercise on healing kinetics early (day 0 through day 5) post-wounding. When RM-ANOVA was performed only on days 0-5, analysis revealed a nearly-significant time × activity interaction (Figure 2C, $F_{2.2,24.1}$=3.229, p=0.053) and a significant main effect of activity (Figure 2C, $F_{1,11}$=4.856, p=.050). Post-hoc analysis revealed significant differences in wound size between HFD-Ex and HFD-Sed mice at day 1, day 4, and day 5 post-wounding (Figure 2C, p<0.05).

Wound Gene and Protein Expression Healing kinetics data described above demonstrated, a) that exercise effects healing of only HFD-fed and not Chow-fed mice and, b) that the major effects of the exercise bout happened early (within 5 days) after the wound was applied. This is similar to previous research in our lab using aged mice (21).

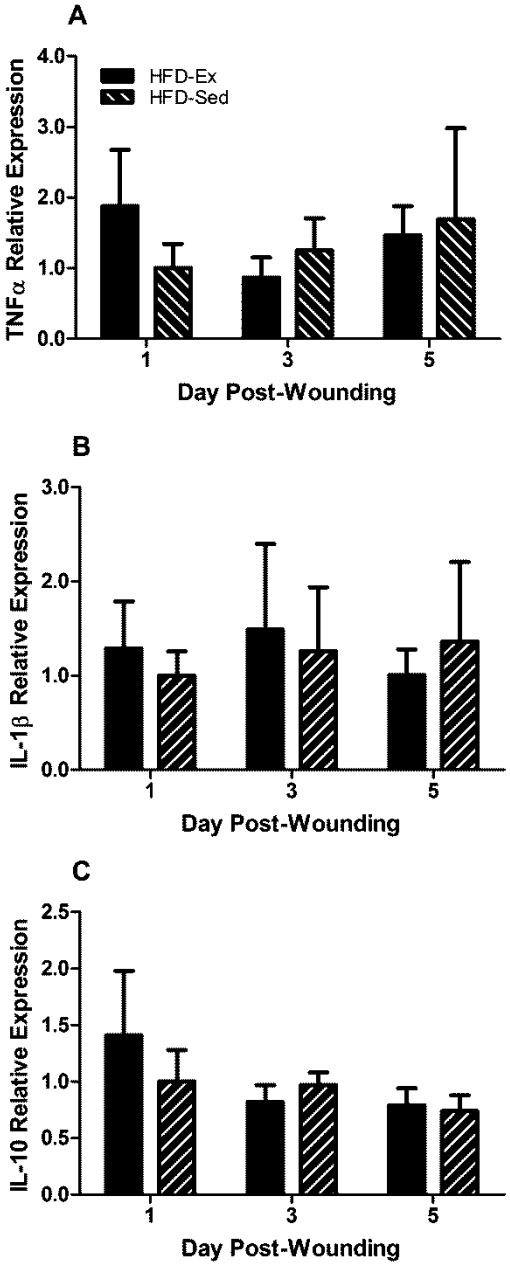

Figure 3: Exercise does not affect cytokine gene expression in cutaneous wounds. **A)** Gene expression of TNF-α. **B)** Gene expression of IL-1β. **C)** Gene expression of IL-10. HFD-Ex: exercised high-fat diet-fed mice (day 1, N=18; day 3, N=8; day 5, N=7). HFD-Sed: sedentary high-fat diet-fed mice (day 1, N=17; day 3, N=8; day 5, N=7). The referent group for each gene is the HFD-Sed, day 1 group. All values are mean ± SEM and were calculated by the $2^{-\Delta\Delta Ct}$ method.

Early responses to wounding consist primarily of inflammation (15), and obesity is known to impair the resolution of the inflammatory state prolonging the healing process (27, 28). Thus, as in our previous study, we focused our analyses on day 1, day 3, and day 5 post-wounding and limited our analyses to HFD-fed mice as exercise did not appear to impact wound healing in Chow-fed mice in any way.

We measured gene and protein expression of pro-inflammatory cytokines IL-1β and TNF-α as well as the anti-inflammatory cytokine IL-10. Previous research has demonstrated that obesity increases levels of pro-inflammatory cytokines and reduces levels of anti-inflammatory cytokines in the wound environment (5, 12, 31, 37). Surprisingly, exercise training did not affect gene expression of any of the cytokine markers measured in this study. There was no day × activity interaction ($F_{2,59}$=1.114, p=0.335) nor main effect ($F_{2,59}$=0.449, p=0.640)

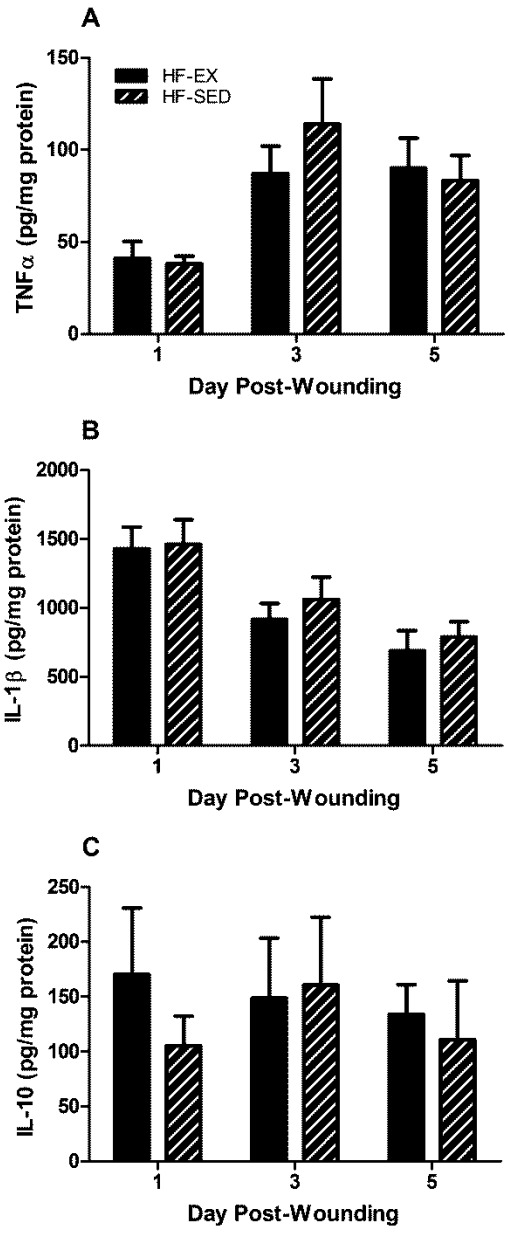

Figure 4: Cytokine protein expression in the wound tissue. **A)** Protein expression of TNF-α. **B)** Protein expression of IL-1β. **C)** Protein expression of IL-10. HFD-Ex: exercised high-fat diet-fed mice (day 1, N=17; day 3, N=8; day 5, N=7). HFD-Sed: sedentary high-fat diet-fed mice (day 1, N=17; day 3, N=8; day 5, N=7). All values are mean ± SEM.

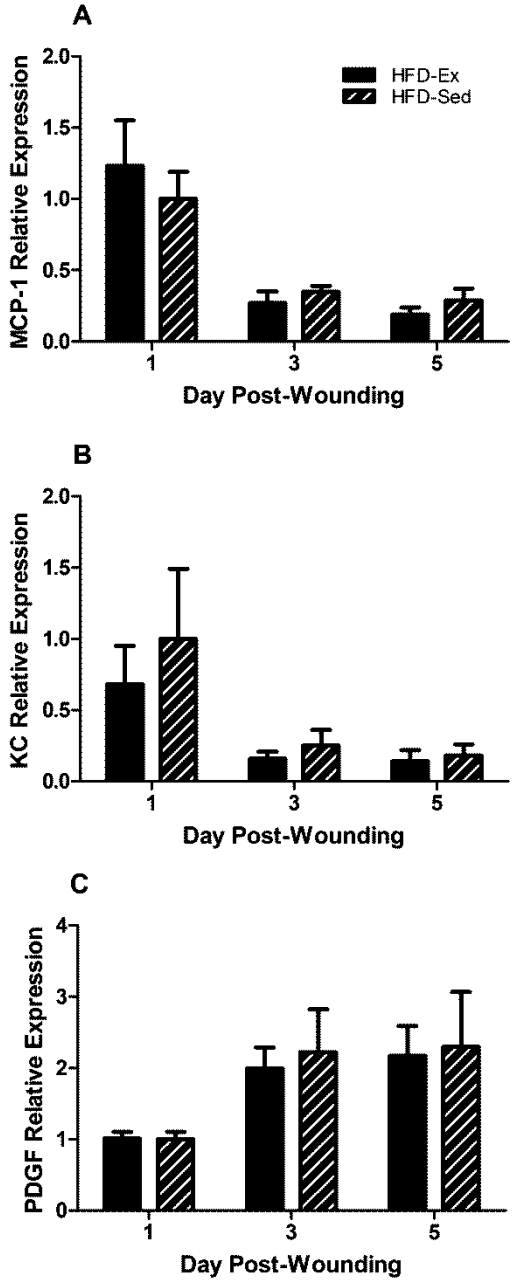

Figure 5: Gene expression of chemokines and platelet-derived growth factor in wound tissue. **A)** Gene expression of MCP-1. **B)** Gene expression of KC. **C)** Gene expression of PDGF. HFD-Sed: sedentary high-fat diet-fed mice (day 1, N=17; day 3, N=8; day 5, N=7). The referent group for each gene is the HFD-Sed, day 1 group. All values are mean ± SEM and were calculated by the $2^{-\Delta\Delta Ct}$ method.

for TNF-α gene expression for activity ($F_{1,59}$=0.042, p=0.839) or day (Figure 3A). Likewise, there was no day × activity interaction ($F_{2,59}$=1.058, p=0.729) nor main effect for activity ($F_{1,59}$=0.022, p=0.882) or day ($F_{2,59}$=0.290, p=0.749) for IL-1β gene expression (Figure 3B). Finally, there was no day × activity interaction ($F_{2,59}$=0.448, p=0.641) nor main effect for activity ($F_{1,59}$=0.711, p=0.139) or day ($F_{2,59}$=1.093, p=0.342) for IL-10 gene expression (Figure 3C).

Although gene expression was unaltered, we hypothesized that post-transcriptional processes might alter the expression of inflammatory proteins. Therefore, we tested wound protein levels of IL-1β, TNF-α, and IL-10 via ELISA at the same time points. Similar to the results of the gene expression assays, exercise training had no effect on protein expression of these cytokines. There was no significant day × activity interaction ($F_{2,58}$=0.921, p=0.404) and no significant main effect for activity ($F_{1,58}$=0.285, p=0.596) for TNF-α (Figure 4A), although there was a significant main effect of day ($F_{2,59}$=15.152, p=0.000). Likewise, there was no significant day × activity interaction ($F_{2,56}$=0.060, p=0.942) and no significant main effect of activity ($F_{1,56}$=0.379, p=0.540) for IL-1β (Figure 4B), although again there was a significant main effect of day ($F_{2,56}$=9.021, p=0.000). Finally, there was no significant day × activity interaction ($F_{2,55}$=0.304, p=0.739) and no significant main effect of either activity ($F_{1,55}$=0.329, p=0.568) or day ($F_{2,55}$=0.150, p=0.861) for IL-10 (Figure 4C).

We additionally hypothesized that exercise might affect influx of inflammatory cells by modulating chemokine expression in the wound tissue. Therefore, we measured gene expression of macrophage chemokine MCP-1 and neutrophil chemokine KC. There was no significant day × activity interaction ($F_{2,57}$=0.470, p=0.627) and no significant main effect for activity ($F_{1,57}$=0.010, p=0.920) for MCP-1 (Figure 5A), although there was a significant main effect of day ($F_{2,57}$=13.039, p=0.000). Likewise, there was no significant day × activity interaction ($F_{2,59}$=0.312, p=0.733) and no significant main effect of activity ($F_{1,59}$=0.796, p=0.376) for KC (Figure 5B), although again there was a significant main effect of day ($F_{2,59}$=8.480, p=0.001).

Because we found no effect of exercise on wound inflammation in obese mice, we hypothesized that exercise might affect other aspects of the early healing response such as hemostasis. Therefore, we tested gene expression of PDGF, a growth factor released upon platelet activation. There was no significant day × activity interaction ($F_{2,59}$=0.091, p=0.913) and no significant main effect of activity ($F_{1,59}$=0.218, p=0.642) for PDGF (Figure 5C). There was a significant main effect of day ($F_{2,59}$=11.859, p=0.000).

DISCUSSION

The major finding of this study was that short-term treadmill exercise (3 days prior-to and 5 days after wounding) speeds wound healing rate in obese, high-fat diet-fed female mice. This is in line with previous studies in aged mice (21) and older adults (3), but to the best of our knowledge this is the first report of an exercise effect on cutaneous wound healing using an obesity model. Obesity is known to impede wound healing (38), and this has been demonstrated in animal models of obesity (32).

As the effect of exercise was seemingly limited to early (day 0 to day 5) post-wounding, we hypothesized that exercise would reduce wound inflammation, which has been previously shown to be excessive in obese mice (5, 12, 31, 37) and has been shown to be ameliorated by exercise in aged mice (21). Surprisingly, unlike our previous study in aged mice, exercise seemed not to affect wound site gene or protein expression of inflammatory cytokines TNF-α and IL-1β or of anti-inflammatory cytokine IL-10 nor wound site gene expression of chemokines MCP-1 and KC. This is to our knowledge the first report of an exercise effect on wound healing which is unrelated to alterations in wound site inflammation. This finding warrants further study.

There are some potential limitations to this study, primary of which is the use of only female mice. This was done to remain consistent with previous studies in aged mice (21) and in restraint-stressed mice (19) as these studies were major sources of inspiration for the study reported here. However, both previous studies used mouse strains different from the C57Bl/6J strain used in this study (Balb/cByJ and SKH-1 mice, respectively). The choice of C57Bl/6J mice was made due to their suitability as a model of diet-induced obesity (36), and this strain of mouse is commonly used in obesity research. It is possible that exercise induces differential effects on wound tissue in these mouse strains, as previous research showed an exercise effect on healing rate which approached significance in young, lean Balb/cByJ mice (21), while we detected no difference in lean C57Bl/6J mice.

In female mice, estrogen plays a major role in protection from obesity-impaired wound healing (18). In the aged mouse study previously performed by our lab (21), the mice were post-menopausal and thus the majority of the estrogen effect was removed. However, our mice were much younger and pre-menopausal, thus estrogen may be playing a protective role in reducing inflammation and speeding healing. This is partially supported by the finding that 6 week old male C57Bl/6J mice heal more slowly than their female counterparts (unpublished data). It is possible that an extension of this study to male mice might demonstrate an exercise effect on healing rate and wound inflammation similar to that seen in post-menopausal female mice in our previous aging study. This is a possibility which needs future study.

Several other potential mechanisms for the exercise effect on wound healing in obese mice require further investigation. Clotting and hemostasis represent the earliest responses to wounding (15), happening generally within 30 minutes post-trauma. Exercise is known to positively influence hemostasis, possibly by increasing activity of coagulation factors as well as by increasing reactivity of platelets (24). Obesity and diabetes are linked to a dysregulated hemostasis through the maintenance of a pro-coagulant state (11). Both obesity and sedentary behavior have been shown to be risk factors for the development of inflammation and hemostatic imbalances (16). Thus, it is possible that exercise-induced alterations in hemostasis may explain the effects of exercise in HFD-fed mice seen in this study, and this potential mechanism should be evaluated. Although we saw no differences in PDGF in this study, it is likely that our first measure at 24h post-healing was taken too late to capture differences in hemostasis as this process generally occurs during the first 30-60 minutes of healing. The increase in PDGF expression seen at days 3 and 5 post-wounding (compared to day 1) in this study likely reflect increased PDGF production by cells such as wound-associated macrophages rather than PDGF production resulting from platelet activation.

A second area which bears consideration for future research is the role of exercise in promoting myofibroblast migration and wound contraction in obese mice. Myofibroblasts are specialized cells that induce wound site contraction and are important in the healing of rodent tissues (15). Treadmill running has been previously shown to

induce migration of myofibroblasts into the patellar tendon of female mice (34), a finding which lends some credence to this hypothesis. Recent evidence indicates that diet-induced obesity causes a delay in myofibroblast differentiation in the wound site of HFD-fed rats (28). Thus, the impact on myofibroblast migration and differentiation may be an important mechanism by which exercise exerts its pro-healing effect in obese, HFD-fed mice.

CONCLUSIONS

This study is the first to demonstrate an effect of exercise on cutaneous wound healing in a model of obesity. Although exercise sped healing rate early after wounding, contrary to previous aging studies, this was seemingly unrelated to alterations in wound inflammation. Future research in this area should focus on the role of early events post-wounding (hemostasis, myofibroblast activity) as well as the differential roles of mouse strain and sex as these are thought to have a major impact on healing kinetics.

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